THE EFFECTS OF DISPERSION YELLOW 3, A WATER POLLUTANT, ON THE DEVELOPMENT OF CHICKEN EMBRYOS

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ZENA MAE DEAS

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ABSTRACT

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The Effects of Dispersion Yellow 3, a Water Pollutant, on the Development of Chicken Embryos

Advisor: Dr. Roy Hunter, Jr.

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The azo dye Dispersion Yellow 3 is used in the carpet industry of Northwest Georgia and is discharged into the Coosa River of the Atlanta, Georgia Region. The present investigation had as its objective to study the effects of DY 3 on the development of chicken embryos. The study proposed to determine if DY 3 induced a characteristic effect in the embryo when given at selected periods of development, and whether DY 3 caused abnormalities in specific organs/tissues of these embryos.

Fertile eggs of White Leghorn chickens were injected with 0.3 cc of 6% solution of Dispersion Yellow 3 for experimental embryos and 0.3 cc of 0.85% solution of saline for control embryos at 48 hr incubation. A total of 758 eggs was utilized in this investigation, with 330 representing the control and 428 representing the experimental.

Macroscopic studies on DY 3-treated embryos from days 6 through 12 revealed anomalies such as hemorrhaging, anopthalmia, micropthalmia, stunted growth, exposed visceral organs and malformation of beaks. Microscopic studies of the dye-treated embryos revealed anomalies such as ruptured

kidney tubular structures and blood vessels, hemorrhaging in mesonephric tubules and destruction of the glomeruli. Distortion in the continuity of the layers (ependymal, mantle, and marginal) surrounding the spinal cavity was also observed. These data have shown that Dispersion Yellow 3 is capable of teratogenic action on chick embryonic development.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Dispersion Yellow 3 (DY 3) is an azo dye that is synthesized from a class of compounds known as diazonium salts. Diazonium salts are synthesized by a series of reactions involving primary amines and nitro compounds (Morrison and Boyd, 1977). The azo dye DY 3 is used in the carpet industry of Northwest Georgia and is discharged into the Coosa River of the Atlanta, Georgia Region. The carpet industry is one of the major users of Georgia's water resources. Tincher (1975) reported that approximately 20 gallons of water are required to process each pound of carpet, with a total of 22 billion gallons required for carpet processing in Georgia in 1974 (943,080,000 square yards x 2 pounds per square yard x 20 gallons per pound x 0.60). Large quantities of organic and inorganic chemicals used in the various carpet manufacturing steps are subsequently being dumped into the Coosa River Basin. The identities and concentrations of the chemical species being dumped into streams are not known; however, the concentrations of carpet manufacturing in the Coosa River Basin have presented considerable concern to officials responsible for water quality in the state of Georgia and its tributaries.

The presence of DY 3 as a water pollutant has aroused concern because it is an azo dye and has been shown to be mutagenic and toxic to microorganisms. Yoshida (1933) demonstrated conclusively the carcinogenicity of an azo dye by showing that 2', 3-dimethyl-4-aminobenzene (O-aminotoluene, AAT; II) induced epithelial proliferation in experimental animals. Upon administering DY 3 orally to the fathead minnow, Tincher (1975) noted

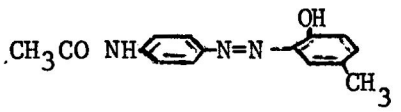
that the chemical had a toxicity of TL 50: 180 mg/L. TL 50 represents the lethal concentration at which 50% of the organisms are killed.

The present investigation had as its objective to study the effects of DY 3 on the development of chicken embryos. The study proposed to determine if DY 3 induced a characteristic effect in the embryo when given at selected periods of development, and whether DY 3 will cause abnormalities in specific organs/tissues of these embryos.

CHAPTER II

REVIEW OF LITERATURE

The mutagenicity of dyes discharged by textile industry represents serious environmental and health hazards due to the toxic properties of these dyes and their adverse effects on water quality. According to Bender (1977) effluent water from the Dalton waste water plant was found to cause frameshift mutations in tester strain TA1537. The Ames test was utilized to examine the mutagenic character of several water supplies and a number of aquatic pollutants. This test was found to be effective as well as useful in identifying certain types of mutagens. Results indicated that the thirteen dyes assayed by Bender represented approximately 65% of the bulk of dyes used in the North Georgia carpet industries. Six carpet dyes, used in high volume in the tufted textile industries of the Dalton area, were found to cause frameshift mutations in the same strain. DY 3 is one of these dyes and has been shown to be mutagenic to microorganisms. The chemical structure of the compound is represented as:



(p-Aminoacetanilide-p-Cresol)

According to the Organic Characterization Study of the Northwest Coosa River Basin conducted in 1974 (EPA Report, 1974) organic compounds that are identified with the tufted textile industry are entering streams in the Coosa River Basin through discharges from waste water treatment plants at Dalton and Calhoun, Georgia. Tincher (1975) reported that the tufted carpet and rug industry in Georgia accounted for approximately 60% of all soft floor coverings produced in the United States, with

approximately 50% of all carpet production in the United States being located in 8 Northwest Georgia counties (Whitfield, Bartow, Coctoosa, Chattooga, Floyd, Gordin, Murray, Walker).

Tincher (1975) has determined spectrophotometrically the quantities of dye in the dye bath before and after the dye cycle. Results indicated that larger quantities of dyes were undoubtedly left in the dye bath, with an average value of 92% exhaustion for disperse dyes on nylon and 78% exhaustion for disperse dyes on polyester. In 1974, Tincher reported a quantity of 978,227 pounds of DY 3 used in carpet piece dyeing, with a total quantity of 17,217 pounds being discharged. Water is the primary recipient of these textile dye effluents. It has been estimated that 3.3 million pounds of disperse dye were used in carpet production in 1974 (139,746 pounds x 10.18 for nylon plus 38,191 pounds x 8.06 for polyester or 46% of all dye used). Of this estimate, 1.3 million pounds of these materials were discharged in 1974, if one would assume that the average disperse dyes contain 40% lignin sulfonate as a diluent and dispersing agent.

Gillman et al. (1948) were the first to report the teratogenic action of an aqueous solution of an azo dye, trypan blue, injected into female rats before and during pregnancy. Some of the abnormalities produced by this type dye were hydrocephalus, rumplessness, eye defects, beak defects, gastroschisis, spina bifida, and malposition of the hind limb. Malformations occurred rarely when injections were given only before pregnancy; occurred in 8.6% of the offsprings when given only during pregnancy; but occurred in 32.4% of the young when given both before and during pregnancy. It has been confirmed by several investigators that treatment only during

pregnancy caused abnormal development in the rat (Hogan et al., 1950), in the mouse (Murakami, 1952; Hamburgh, 1952, 1954; Waddington and Carter, 1952), and in the rabbit (Harm, 1954).

Wilson (1954) reported the teratogenic activity of fourteen azo dyes that are chemically related to trypan blue for their effect on pregnant rats. The dyes are Evans blue, Niagra blue 4B, Niagra sky blue 6B, Congo red 4B, Dianil blue 2R, Erie violet 2B, Erie garnet B, Niagra blue 3RD, and azo blue. Trypan blue was the most potent, causing malformations most frequently in the brain, vertebral column, heart and major arteries, and the eye. Several other structures were occasionally affected. Somewhat less effective were Evans blue which caused abnormality in 14%, Niagra blue 4B in 4%, and Niagra sky blue 6B in 3%. The remaining dyes did not show any noticeable activity. The brain was the organ most sensitive to the teratogenic action of azo dyes. Approximately 80% of the brain defects after trypan blue, and all brain defects after the other dyes were in the form of internal hydrocephalus. Histological studies in hydrocephalic animals revealed that the mesencephalic aqueduct was either obliterated or reduced in size. The cause of reduction was not apparent; no evidence of undue proliferation in the vicinity of the aqueduct was found. Other less frequent abnormalities of the brain were meningocele, meningocephalocele, and exencephaly. Ocular anomalies, although less frequent, were often associated with brain abnormalities. Anopthalmia accounted for about half of the ocular defects, was rarely bilateral, and occurred more often on the left than on the right side.

Callaway (1976) used trypan blue in her studies and observed that some of the abnormalities produced in chicken embryos were rumplessness, growth retardation from crown to rump, micropthalmia, and red hematomas. A high incidence of rumplessness was prominent throughout her investigation. Waddington and Perry (1956) have reported the teratogenic action of trypan blue on amphibian embryos. The malformations observed involved primarily the neural axis, skeleton, heart, and major blood vessels.

Increasing amounts of waste are being directed to the water for disposal. At the same time, there is increasing evidence of environmental damage resulting from improper operation. Serious environmental hazards are created by effluents from textile mill dye houses and carpet manufacturing because they contain significant amounts of various heavy metals. According to Netzer et al. (1975) many of these wastes contain a substantial concentration of soluble organics, suspended solids and dissolved salts. Chromium, lead, copper, mercury, and nickel are the most prevalent metals in dye effluents. Zinc, copper, and chromium are present as an integral part of numerous dyes.

In many industrial (including carpeting) effluents, lead is a significant water pollutant because of its indiscriminate release. The effect of lead on the early development of the chicken embryo specifically disturbed the morphogenesis of the central nervous system (Catizone and Gray, 1941). Cherry (1976) observed that brain hemorrhaging was the most frequent anomaly associated with the use of lead acetate. Dwarfism and micropthalmia were also observed.

Singh et al. (1977) have investigated the accumulation of heavy metals in the rainbow trout, Salmo gairdneri, fed on a diet containing 30% sewage sludge for 10 weeks. Their results indicated that rainbow trout accumulated only a small proportion of the heavy metals they ingested. The accumulations seem to some extent to have been independent of the actual amounts consumed. Iron was the most concentrated heavy metal in the experimental diet yet it showed only a 22.3% increase in the experimental group at the end of the 70 day period. Manganese, the second most concentrated metal, showed no accumulation at all. In the experimental fish, the nickel concentration was 3.5 times greater, but at the end of the experiment 90.9% more nickel was present in the experimental fish. Significant accumulation of chromium occurred although the chromium concentration was not high in the experimental diet. According to Fromm et al. (1962), rainbow trout are known to accumulate chromium from water containing as little as 1 mg 1000 l^{-1} of this metal as chromate. The extent to which heavy metals were absorbed through the gut wall depended upon their precise chemical form (Bryan, 1976). There were no significant changes in the body levels of experimental rainbow trout when exposed to manganese, cobalt, copper, and cadmium. Chromium and lead both showed a rise followed by a significant reduction in concentration; whereas, the increase in zinc and nickel is the most likely to give rise to problems because of their adverse toxicity.

Watter et al. (1965) investigated rose bengal chromoexcretion, a property of the parenchymal cells in rats during feeding of 4-dimethylaminoazobenzene, a carcinogen, and 2-methyl-4-dimethylaminoazobenzene (2-Me-DAB), a noncarcinogen. Azo dye hepatocarcinogenesis involves the parenchymal cells.

Carcinogenic and noncarcinogenic azo dyes behaved differently toward rose bengal uptake by the liver. The carcinogenic azo dye p-dimethylaminoazobenzene (DAB) decreased to a great extent the rose bengal uptake, whereas, the noncarcinogenic azo dye 2-Me-DAB produced no change. Abnormal liver function after DAB feeding is related to the carcinogenic effect of DAB. It has been shown that progressive degeneration of liver parenchyma occurs during the first 30 days of azo dye hepatocarcinogenesis (Orr, 1948). The effect of DAB on liver function could therefore be attributed to early damage of parenchymal cells, since rose bengal uptake and excretion required a normal functioning parenchyma. Moreover, it was shown that hypobasophilia and necrosis modified the morphology of liver. During azo dye hepatocarcinogenesis, important changes have been observed in the liver. According to Daoust et al. (1959) there is a reduction in the parenchymal cell population; decreased basophilia (Opie, 1946), as well as degenerative changes followed by necrosis (Price et al., 1952). These morphologic changes were found to originate in the region of the central vein.

The kinetics of biliary excretion of some azo dyes have been examined in the rat. The protein binding of each dye to blood and liver in vitro was examined. The dyes appeared in the biliary cannula within 3-5 min of injection. The kinetic analysis of results obtained with the dyes after a dose of 20 moles indicated that the excretion process may be described by two first order processes, an initial rapid excretion of most of the dye followed by a slower residual excretion. It can be concluded from the dyes studied that their rate of biliary excretion appears to be a function of their relative degree of binding to liver proteins as against blood proteins. The dye-protein binding in the liver is an integral part of the

excretion process and not a storage phase delaying excretion (Andrews et al., 1961).

Fare (1966) reported that rats "painted" with certain azo dyes (amino-azobenzene and 4-monomethylaminoazobenzene) dissolved in acetone developed multiple skin tumors of histologic types. Tumors of the ear duct arose only in rats treated with any one of the 3-methoxy dyes. These tumors were invariably unilateral. Azo dyes unsubstituted in the 3-position with a methoxy group are carcinogenic to rat skin.

Gray (1978) investigated the effects of Dispersion Yellos 3 (DY 3) on oocyte development in Rana clamitans larvae. Her results indicated that there was a decrease in intramitochondrial inclusions, an increase in membranous and vesicular materials and dissociation between the follicle cells and the surface of the oocyte. There was a breakdown of the follicular membrane and oolemma which preceded the deposition of the extra-nuclear materials. Aberrations observed in chromosomes from regenerating tail squashes suggested that the dye had a toxic effect, leading to gaps, breaks, dicentrics, and rings (Gray et al., 1979). Edema of the body cavity was the most frequently observed morphological change. A similar change was noted by Levine (1932) in Rana clamitans larvae exposed to X-rays.

Upon examining the effects of the azo dye Acid Yellow 135 in the fertile eggs of White Leghorn chickens, Wright (1977) observed that this dye produced hemorrhaging, micropthalmia, anopthalmia, stunted growth, exposed viscera and feather inhibition. Histological analysis revealed that cellular necrosis was present in the lens fibers and around its periphery, producing an abnormal appearance of lens epithelium. Hemorrhaging was

detected in the kidney and liver. Harvey (1979) observed necrosis of the retina, irregularity in the shape of the lens and cells of the lens epithelium, absence of the cornea, hemorrhaging in the cavity of the diencephalon, the appearance of a second lens behind the optic nerve.

Heavy metals as pollutants are generally discharged as a result of industrial process and are a major problem because of their toxicity. The possible long term health effects of dyes and dye degradation products are becoming of increasing concern. More research should be directed toward the area of textile industrial pollution since it is a relatively new area of establishing mutagenicity in organisms. The possible mutagenic, carcinogenic, and/or teratogenic effects of dyes should be subjected to extensive tests in a variety of animals since man is exposed to mutagens in food and drinking water.

CHAPTER III

MATERIALS AND METHODS

Source of Material

Fertile eggs of White Leghorn chickens were obtained from Henson Rabbitry in Bowdon, Georgia. They were maintained at a temperature of 37.8 C. All eggs were swabbed with 70% alcohol prior to injection to prevent contamination. The azo dye Dispersion Yellow 3, a water pollutant, was injected into the experimental eggs. Dispersion Yellow 3 was obtained in powdered form from Dr. Wayne Tincher, School of Textile Engineering, Georgia Institute of Technology.

Injection Procedure

For preliminary studies, DY 3 was used at concentrations of 4 mg/ml, 1%, 2%, 4%, and 6% in 0.85% saline. Groups of six to twelve eggs were injected with increasing dosages, from 0.2 cc to 0.9 cc, of each concentration. In many instances, several confirmatory runs were necessary to obtain a reasonably consistent picture. These different concentrations were used to determine the tolerance level and whether any gross effects were produced. The final concentration used in all subsequent experimentation was 6%. The dosage that was selected to be used throughout this investigation was 0.3 cc.

The test agent (DY 3) was injected after 48 hr of incubation. Experimental eggs were injected with 0.3 cc of a 6% solution of Dispersion Yellow 3. Control eggs were injected with 0.3 cc of 0.85% sterile saline. The total number of eggs used in this investigation was 758. Using a sterile syringe, injections were made through the hole drilled through the air

sac in the blunt end of the egg. Following injections, the eggs were sealed with tape and returned to the incubator for further development. The embryos were observed after 6-12 days of incubation and examined grossly for the number and kinds of abnormalities produced. Photographs were taken of selected embryos using the Kodak Ektagraphic Visualmaker.

Histological Procedure

For histological examination, the embryos were fixed in 10% buffered formalin. In preparation for paraffin embedding, the embryos were dehydrated in a series of 50%, 70%, 80%, 95%, and 100% ethanol at 1 hr intervals each, with 2 changes of 100% ethanol. They were then transferred to a 50:50 solution of toluene and 100% ethanol for 1 hr, with two 30 min changes, and cleared in toluene for 1 hr. Infiltration was in toluene/paraffin at 1 hr intervals each and then 1½-2 hr in paraplast; and embryos were embedded in paraplast. Sections were cut at 9 microns on the 820 Spencer Rotary Microtome, positioned on albumenized slides, and allowed to dry on a slide warmer.

The hematoxylin/eosin general staining method was used. Slides were treated as follows:

1. Deparaffinized in xylene and rehydrated in descending concentrations of ethanol 2-3 min each
2. Washed in running water 3-5 min
3. Stained in Harris' hematoxylin 3-5 min
4. Washed in running water 3-5 min
5. Placed in Scott solution 3-5 min
6. Washed in running water 3-5 min
7. Counterstained with 0.5% eosin in 70% ethanol 2 min

8. Dehydrated in ascending concentrations of ethanol 1-3 min
9. Cleared in xylene
10. Mounted in Kleermount

Stained slides were studied and photographs of selected sections were taken using the American Optical Microscope ExpoStar Photomicrographic Assembly.

CHAPTER IV

EXPERIMENTAL RESULTS

Some effects of DY 3, a water pollutant, on developing chicken embryos have been determined. The results will be presented under preliminary studies, macroscopical and microscopical observations.

Preliminary Studies

Preliminary studies determined the tolerance level and gross effects produced. Concentrations ranging from 6% to 4 mg/ml of DY 3 were used. Tables 1 and 2 summarize the results of exposing embryos to various concentrations and dosages in order to arrive at the final concentration used in this study (0.3 cc of a 6% DY 3). The dose producing abnormalities varied with the age of the embryo at the time of injection. All final injections were performed after 48 hr of incubation.

When the experimental embryos were injected with 0.6 cc of a 4 mg/ml solution of Dispersion Yellow 3, a high mortality rate resulted. However, when the dosage was lowered, the level of viability was slightly increased, but the types of malformations were less than 22%. When the concentration of Dispersion Yellow 3 was increased to 1%, there was an increase in malformations. A slight amount of hemorrhaging, growth retardation and inhibition of eye development (micropthalmia and anopthalmia) was observed. At 0.6 cc-0.9 cc, the mortality rate was high (63%). When the concentration of Dispersion Yellow was increased to 2%, there was a slight increase in the viability of the embryos but only a small percentage showed malformations. When the concentration of Dispersion Yellow 3 was increased to 4%, malformations of the embryos increased to 30%, but less than 50% of the

Table 1. Preliminary study on the effects of Dispersion Yellow 3 on chicken embryos injected after 48 hr of incubation.

Conc. mg/ml	(DY 3) %	Dosage cc	Age Days	% Viable	% Malformed	Types of Malformations (%)			
						Eye Inhib.	Hemor-rhaging	Stunted Growth	Exposed Viscera
4		0.2	7	50	50	0	25	15	10
4		0.3	8	67	0	0	0	0	0
4		0.5	8	57	43	0	21	14	7
4		0.6	10	29	0	0	0	0	0
4		0.7	10	57	7	7	0	0	0
4		0.9	7	83	0	0	0	0	0
	1	0.2	7	50	38	4	17	12	4
	1	0.3	8	33	33	33	0	0	0
	1	0.5	8	17	17	0	0	0	17
	1	0.6	10	42	67	17	33	17	0
	1	0.7	10	20	10	0	10	0	0
	1	0.9	7	10	10	0	10	0	0
	2	0.2	7	83	50	2	25	15	8
	2	0.3	8	67	67	33	17	17	0
	2	0.5	8	67	33	0	33	0	0
	2	0.6	10	44	33	0	12	12	6
	2	0.7	10	67	44	7	17	9	11
	2	0.9	7	53	40	0	13	13	13
	4	0.2	7	83	50	4	21	17	8
	4	0.3	8	58	25	5	7	5	8
	4	0.5	8	58	52	10	8	8	17
	4	0.6	10	56	44	11	6	17	11
	4	0.7	10	17	58	0	17	25	17
	4	0.9	7	17	6	0	6	0	0
	6	0.2	7	50	83	3	37	10	33
	6	0.3	8	81	50	19	13	6	13

Table 1. Continued.

Conc. (DY 3) mg/ml	%	Dosage cc	Age Days	% Viable	% Malformed	Types of Malformations (%) Eye Inhib.	Hemor-rhaging	Stunted Growth	Exposed Viscera
	6	0.5	8	55	50	0	25	5	25
	6	0.6	10	44	44	0	22	22	0
	6	0.7	10	17	17	0	17	0	0
	6	0.9	7	17	17	4	8	2	3

Table 2. Preliminary study of saline (0.85%) control embryos injected after 48 hr of incubation.

Dosage cc	Age Days	% Viable	% Malformed	Types of Malformations (%)			
				Eye Inhib.	Hemor-rhaging	Stunted Growth	Exposed Viscera
0.2	7	56	0	0	0	0	0
0.3	8	85	0	0	0	0	0
0.5	8	26	0	0	0	0	0
0.6	8	25	0	0	0	0	0
0.7	8	20	44	8	20	16	0
0.6	10	58	50	0	8	17	25
0.7	10	67	27	0	13	7	7
0.9	10	25	26	0	20	0	0

embryos were viable. Administration of a 0.7 cc dosage resulted in a high mortality rate (75%). At 0.6 cc, the mortality rate decreased slightly, with a slight increase in the number and type of malformations.

When the concentration of Dispersion Yellow 3 was increased to 6%, several types of malformations were observed at different dosage levels. In many instances, however, the dye appeared to be generally toxic and the embryos either died, or, if they survived, there was no effect. Three embryos died during the first six days and 3 of them showed extensive hemorrhaging throughout their bodies. Of the 9 embryos sacrificed during the same period, only 2 showed brain hemorrhaging, suggesting that early death was directly associated with brain injury of the embryo. All of 3 embryos dying during the 8 to 12 days of incubation showed brain hemorrhaging. Fifty-three embryos were sacrificed at 8 days; of these 12 showed inhibition of eye development, 7 showed exposed viscera in addition to hemorrhaging, and 3 appeared unaffected. It is apparent that the most common effect was hemorrhaging, seen in 23 out of 30 abnormal experimental embryos, and also in 11 of 22 abnormal control embryos. Also observed but less frequently was micropthalmia. Deficient beaks occurred in less than 15% of the experimental embryos. In addition, controls were injected with 0.85% saline (Table 2). The abnormalities common to both the experimental and control embryos were inhibition of the eye, hemorrhaging, stunted growth, and exposed viscera.

Some dyes induce a characteristic effect on embryos when given at the proper period of development, and the type and frequency of malformations which resulted from these studies were somewhat dependent upon the dosage administered to the experimental embryos.

Macroscopical Observations

The viability of all embryos injected was less than 50% of the total population. The percentage of viability in the experimentals was slightly less than in the controls. Data from DY 3-treated embryos are detailed in Tables 1 and 3.

The DY 3-treated embryos were examined grossly at various days during development (Table 3). Macroscopical observations among experimental embryos included hemorrhaging, inhibition of eye development (i.e., unilateral micropthalmia and anopthalmia), protrusion of visceral organs, stunted growth, deficient beak, and discoloration of the eye. The types of malformations increased significantly in those embryos examined after 6 days. Hemorrhaging, deficient beak, stunted growth (Figs. 1-4, 7-8) and discoloration of the eye (Figs. 9-10) occurred in many of these embryos. The 8-day experimental embryo in Fig. 6 exhibited hemorrhaging and protrusion of visceral organs, in comparison to the control (Fig. 5). Of the abnormalities observed in DY 3-treated embryos, hemorrhaging and protrusion of visceral organs were prominent (Figs. 6, 11-12, 18), increasing steadily from day 6 to day 12 in comparison to the control (Figs. 6, 17) which developed normally. Embryos dying or sacrificed on day 6 exhibited inhibition of eye development (anopthalmia), as seen in Figs. 13, 16, 19-20. Normal development of a control embryo is represented in Fig. 14A in comparison to an experimental embryo shown in Fig. 14B exhibiting unilateral micropthalmia. Unilateral inhibition in eye development (Figs. 15, 22) and protrusion of visceral organs (Fig. 16) were also noted. The control in Fig. 21 represents normal development of the embryo at the 10-day developmental stage.

Table 3. Effect of exposure of 48 hr chick embryos to Dispersion Yellow 3 at various stages of development.

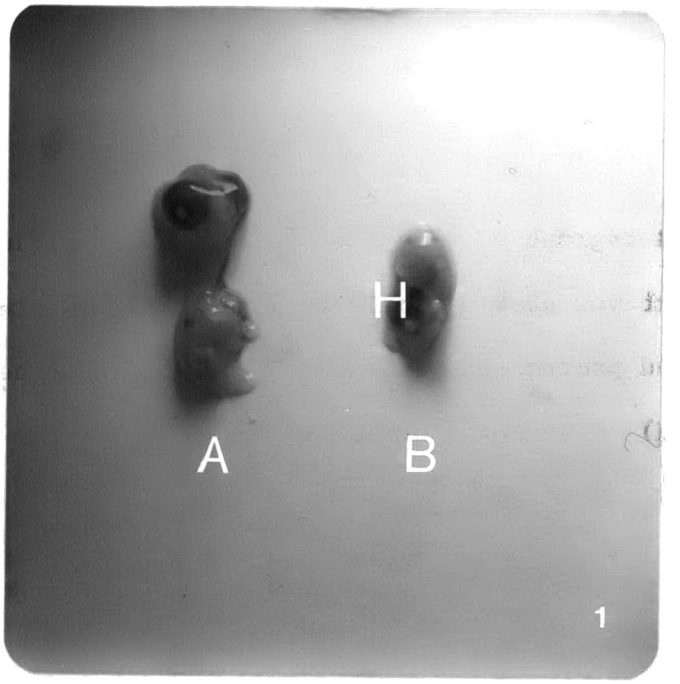
No. Inj.	Age Days	No. (%) Viable	No. (%) Malformed	Types of Malformations (%)				
				Eye Inhib.	Hemor-rhaging	Exposed Viscera	Stunted Growth	Deficient Beak
Controls[a]								
10	6	6 (60)	0	0	0	0	0	0
25	7	22 (88)	0	0	0	0	0	0
35	8	30 (86)	2 (6)	0	6	0	0	0
12	9	9 (75)	0	0	0	0	0	0
27	10	25 (93)	1 (4)	0	4	0	0	0
14	11	5 (36)	0	0	0	0	0	0
12	12	6 (50)	1 (8)	0	0	0	8	0
Experimentals[b]								
12	6	9 (75)	7 (58)	3	42	13	0	0
12	7	10 (83)	8 (67)	8	25	17	17	0
53	8	46 (87)	42 (79)	23	19	3	19	15
10	9	5 (50)	0	0	0	0	0	0
24	10	20 (80)	15 (63)	13	25	13	8	4
6	11	1 (17)	4 (67)	0	33	17	17	0
12	12	7 (58)	6 (50)	0	25	8	17	0

[a]All embryos were injected with 0.3 cc of 0.85% saline.

[b]All embryos were injected with 0.3 cc of 6% Dispersion Yellow 3.

Fig. 1. Photograph of 7-day control (A) and experimental (B) embryos showing unilateral anopthalmia, stunted growth, and protrusion of visceral organs. Note hemorrhaging (H) of visceral organ.

Fig. 2. Photograph of 7-day control (A) and experimental (B) embryos showing growth retardation.


H
A
B
1

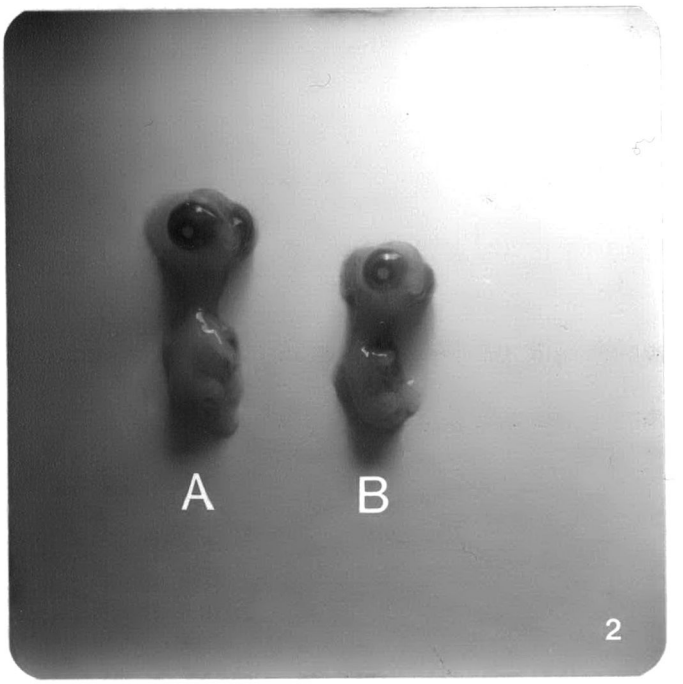

A
B
2

Fig. 3. Photograph of 7-day control (A) and experimental (B) embryos. Experimental embryo shows growth retardation and incomplete body development.

Fig. 4. Photograph of 8-day control (A) and experimental (B) embryos. Experimental embryo shows discoloration in the eye, deficient beak, and stunted growth.


A
B
3

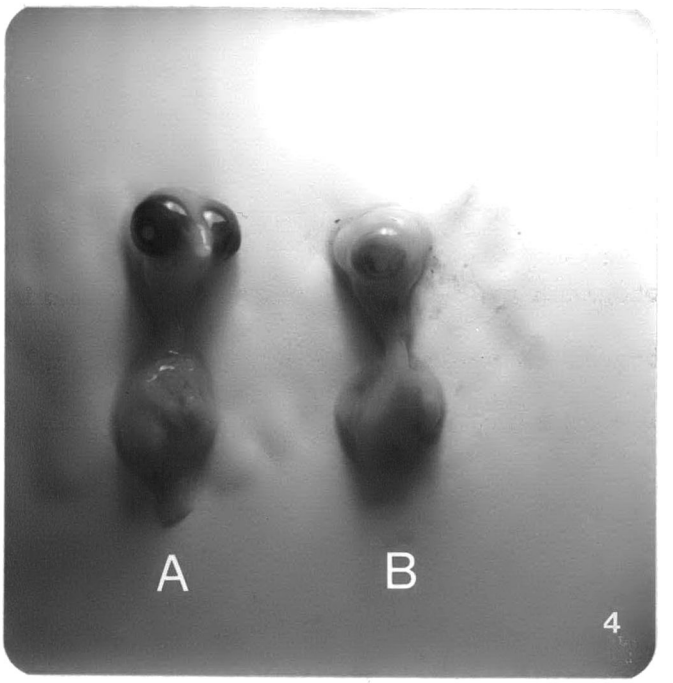

A
B
4

Fig. 5. Photograph of 8-day control.

Fig. 6. Photograph of 8-day experimental embryo showing hemorrhaging (H) throughout the body. Note visceral organs (V) have protruded outside of embryo.


5

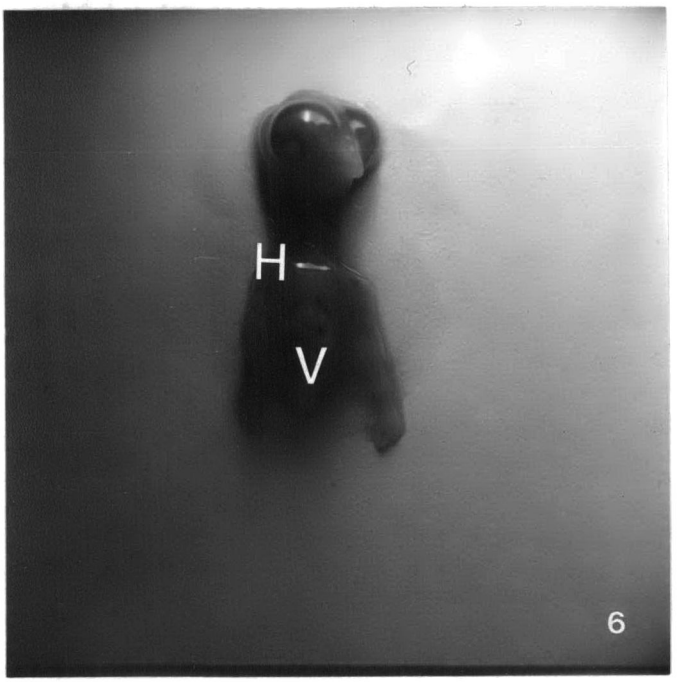
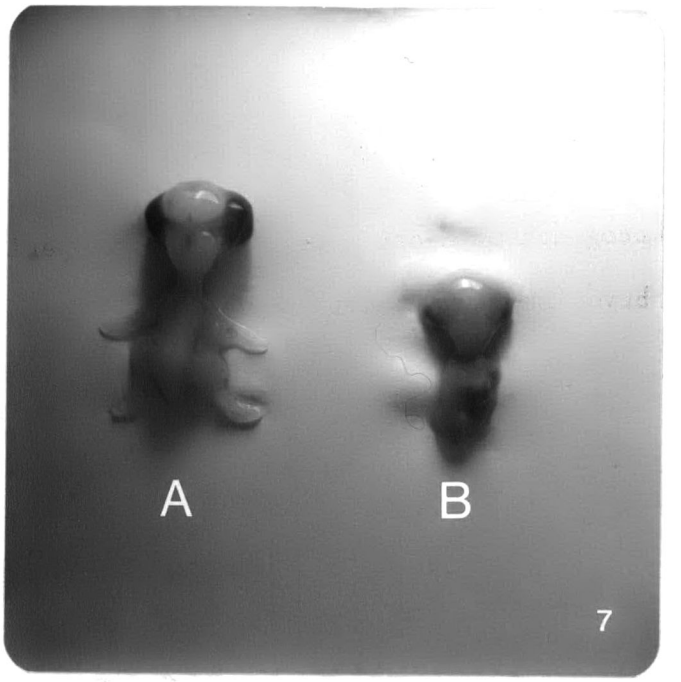

H
V
6

Fig. 7. Photograph of 8-day control (A) and experimental (B) embryos showing stunted growth. Note normal development of the head.

fig. 8. Photograph of 8-day control (A) and experimental (B) embryos showing protrusion of visceral organ (V), deficient beak, stunted growth, and retardation in eye development.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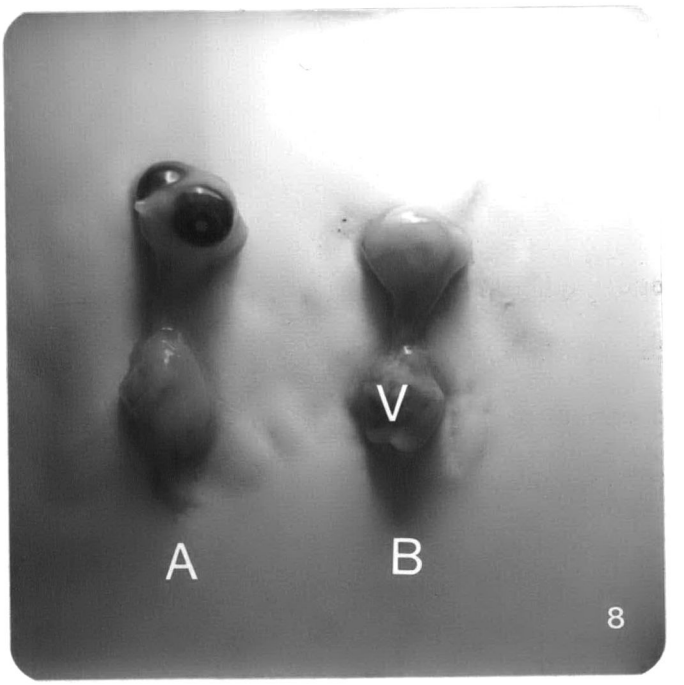
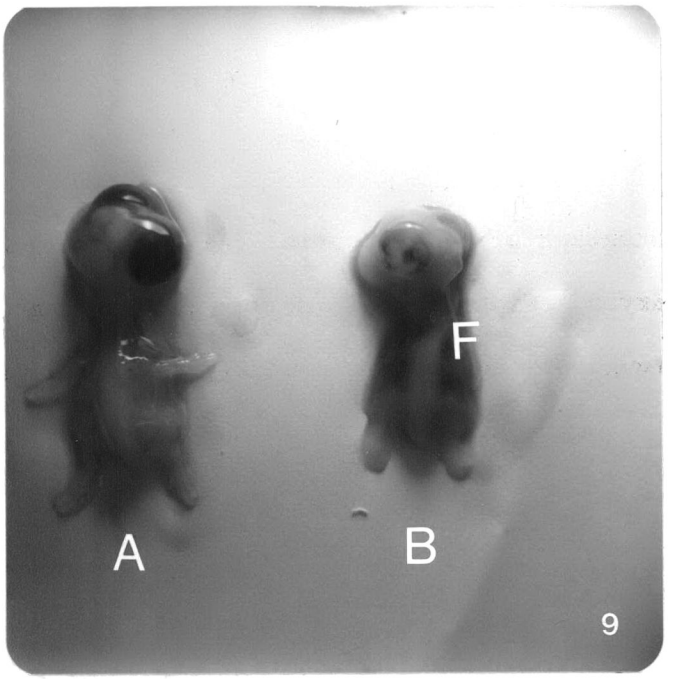

V
A
B
8

Fig. 9. Photograph of 8-day control (A) and experimental (B) embryos. Experimental embryo exhibits hemorrhaging throughout the body, especially the forelimbs (F).

Fig. 10. Photograph of 8-day control (A) and experimental (B) embryos showing discoloration in eye development.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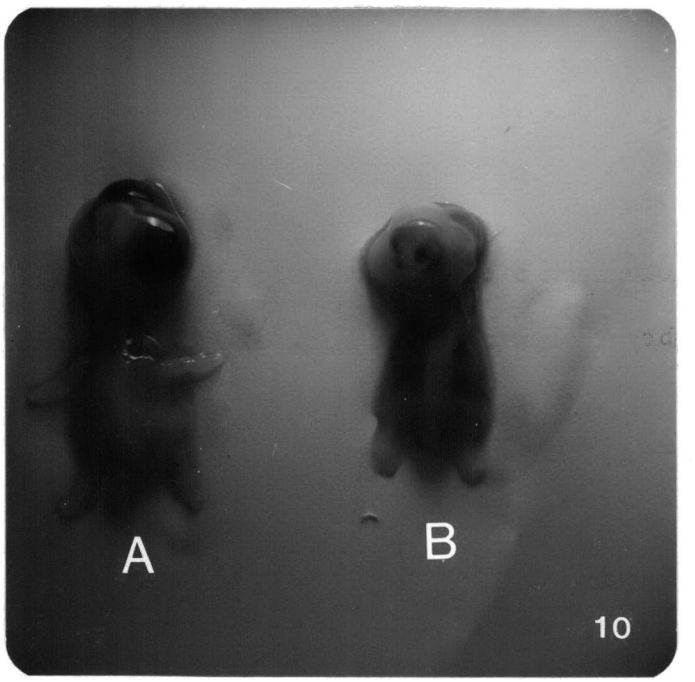
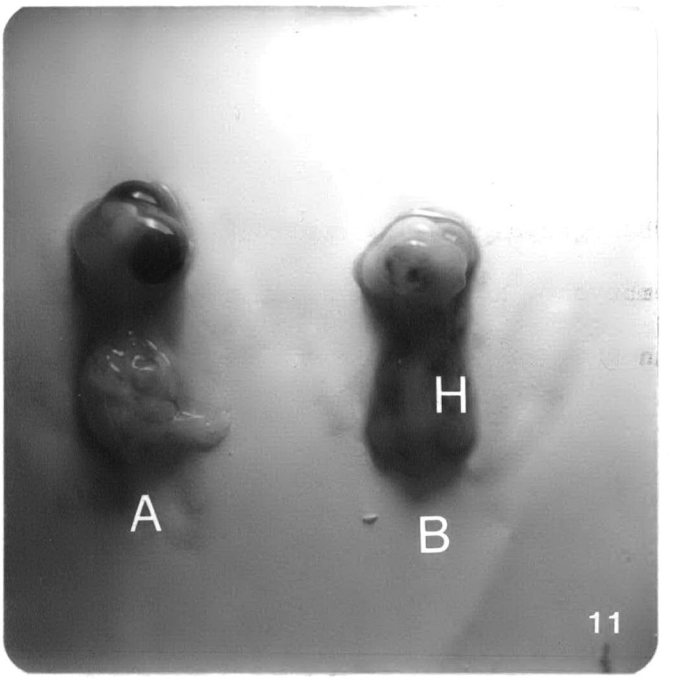

A
B
10

Fig. 11. Photograph of 8-day control (A) and experimental (B) embryos. Note the hemorrhaging (H) and stunted growth in the experimental embryo.

Fig. 12. Photograph of 8-day control (A) and experimental (B) embryos. Experimental embryo showing protrusion of the visceral.


H
A
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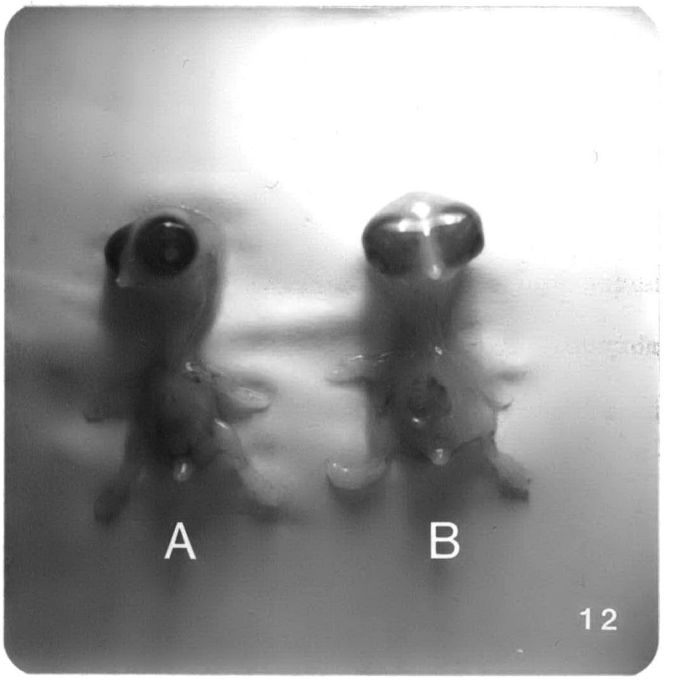
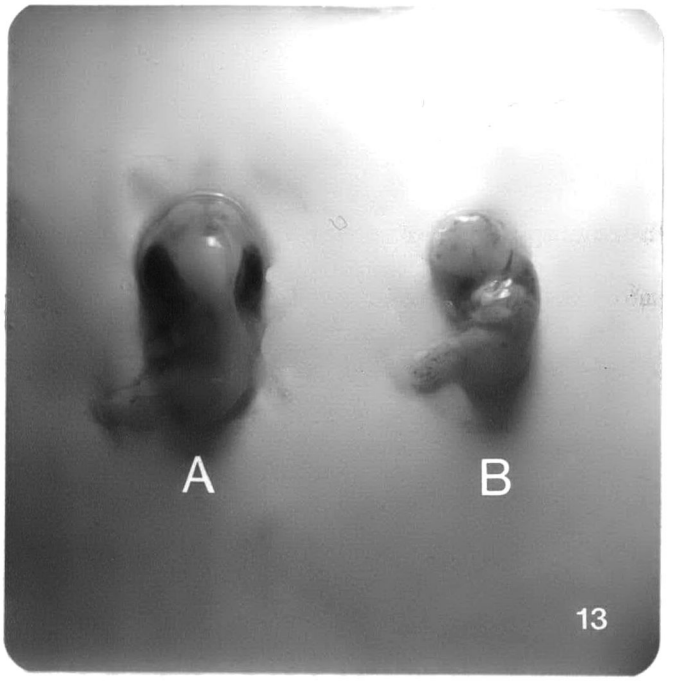

A
B
12

Fig. 13. Photograph of 8-day control (A) and experimental (B) embryos. Experimental embryo exhibits bilateral anopthalmia (eyelessness) and stunted growth.

Fig. 14. Photograph of 8-day control (A) and experimental (B) embryos showing unilateral micropthalmia.


A
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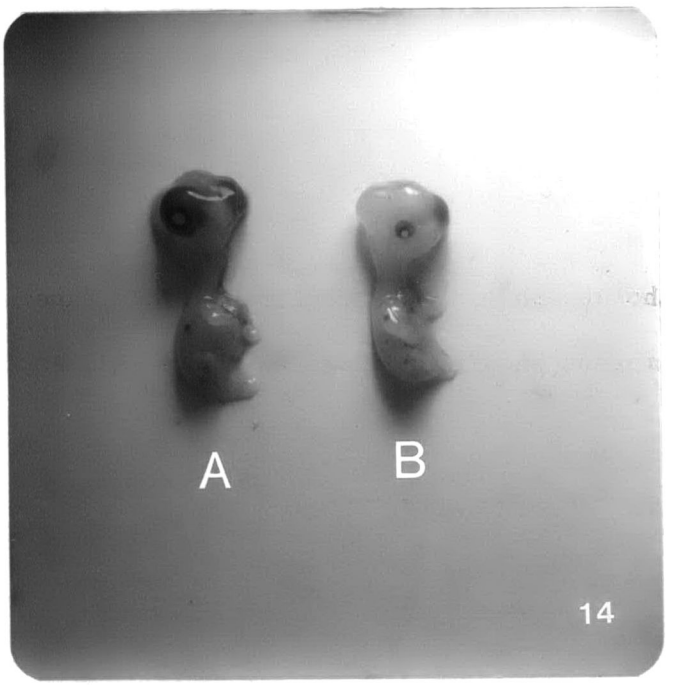
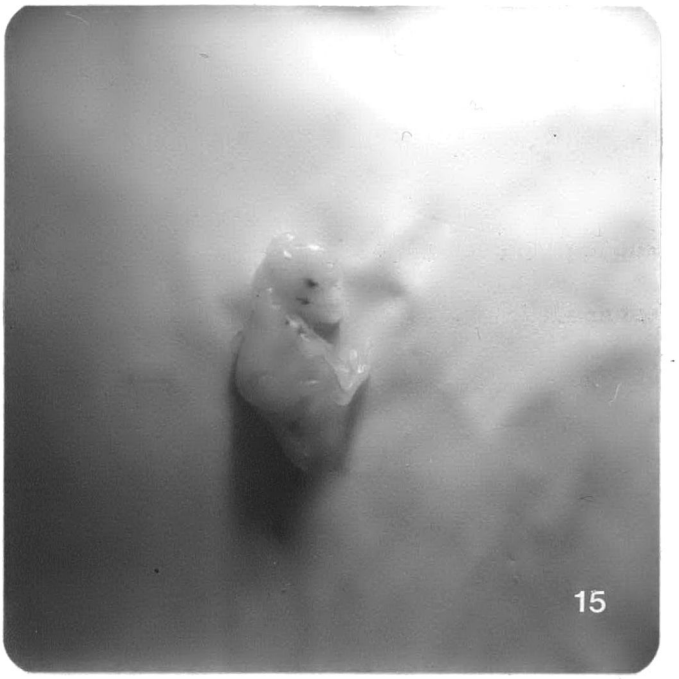

A
B
14

Fig. 15. Photograph of 8-day experimental embryo showing unilateral inhibition of eye development and protrusion of the brain.

Fig. 16. Photograph of 8-day experimental embryo exhibiting anopthalmia and stunted growth. Note the protrusion of visceral organ (V).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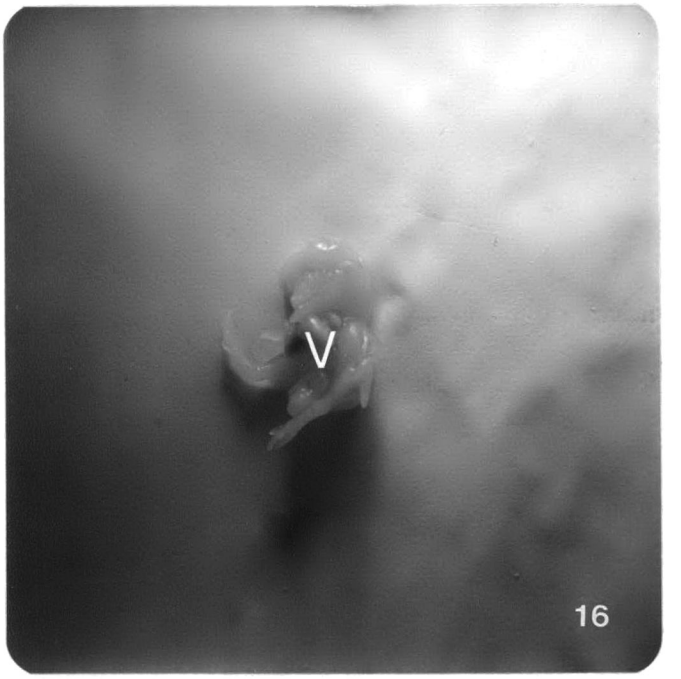

V
16

Fig. 17. Photograph of 10-day control embryo.

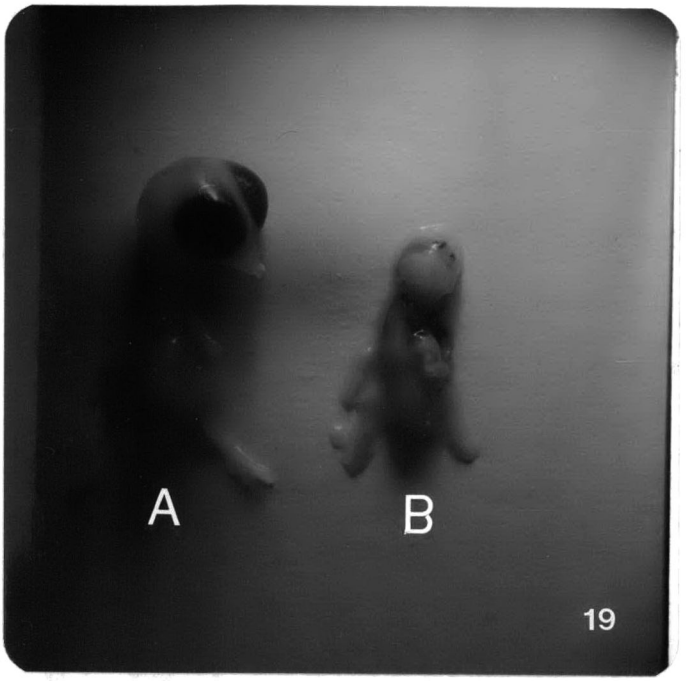
Fig. 18. Photograph of 10-day experimental embryo showing hemorrhaging from the dorsal view.


17


18

Fig. 19. Photograph of 10-day control (A) and experimental (B) embryos showing retardation in eye development (anopthalmia) and stunted growth.

Fig. 20. Photograph of 10-day control (A) and experimental (B) embryos. Experimental embryo exhibited anopthalmia, twisted beak and stunted growth. Note protrusion of brain (C).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B
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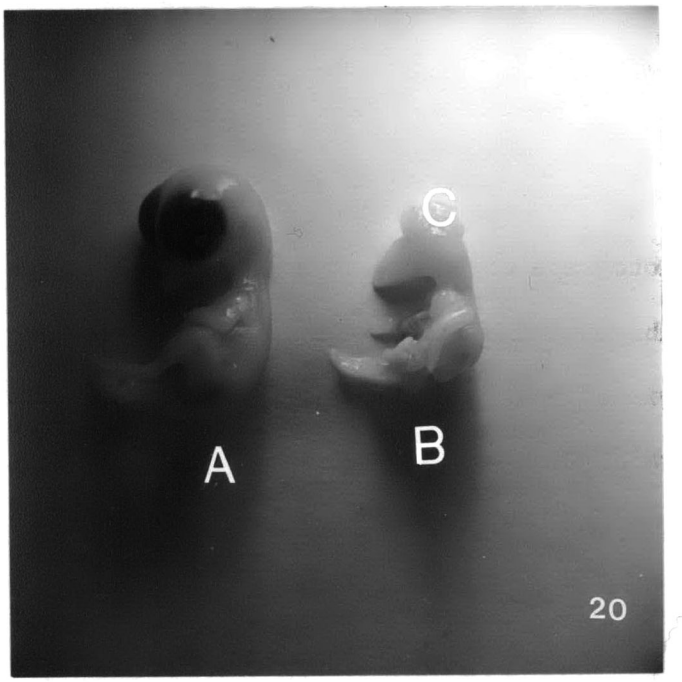

C
A
B
20

Fig. 21. Photograph of 10-day control embryo.

Fig. 22. Photograph of 10-day experimental embryo exhibiting unilateral micropthalmia.


21


22

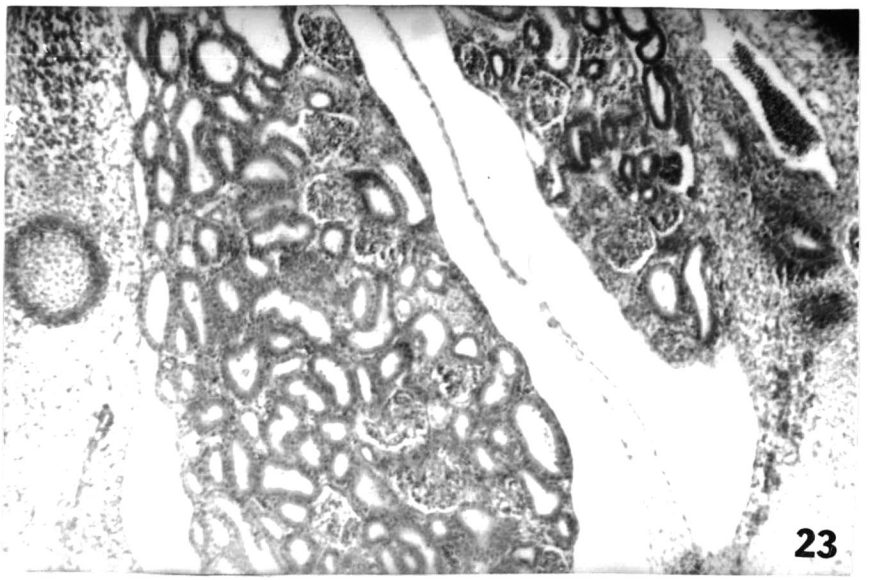
Microscopical Observations

Histological examination of 7, 8 and 10-day DY 3-treated embryos showed hemorrhage in mesonephros tubules, destruction of the glomeruli, ruptured tubular structures and blood vessels, discontinuity in mesonephric tubules, distortion in the continuity of the layers (ependymal, mantle and marginal) surrounding the spinal cavity and hemorrhaging among kidney tubules. Cross sections of 7-day treated embryos showed hemorrhage in the mesonephros as seen in Fig. 24. A control is shown in Fig. 23. Destruction of glomeruli (Figs. 26, 28) was also noted. In controls, glomeruli were well preserved, as shown in Figs. 25, 27. Occasionally, mesonephros has a portion of tubular structure ruptured (Figs. 30, 36, 38, 42, 44) and ruptured blood vessels were also recognized. Hemorrhaging among kidney tubules (Figs. 36, 38, 43-44) was also noted. The absence of hemorrhaging and ruptured kidney tubules is shown in controls, Figs. 29, 37. In some instances, discontinuity in the tubular structures (Figs. 32, 36) occurred. A control is seen in Fig. 31.

There was distortion (Fig. 34) in the continuity of the layers (ependymal, mantle and marginal) surrounding the spinal cavity (see control, Figs. 33, 35). It was also observed that packed blood vessels prominently occurred on one side of the embryo (Fig. 42). Intact tubular structures are shown in controls, Figs. 39, 41.

Fig. 23. Cross section through the mesonephric kidney of 7-day control embryo. 10X.

Fig. 24. Cross section through the mesonephros of 7-day Dispersion Yellow 3-treated embryo showing hemorrhaging (arrow) in the mesonephros. 10X.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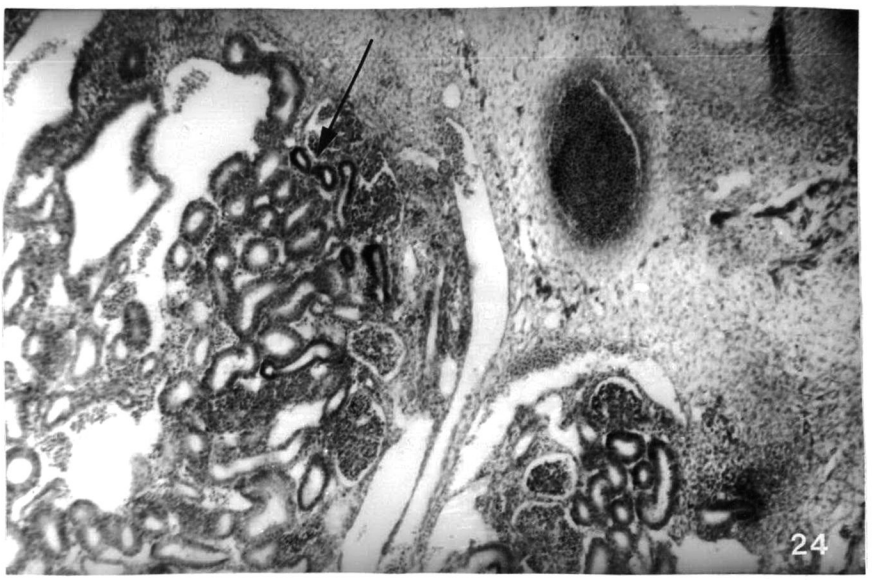
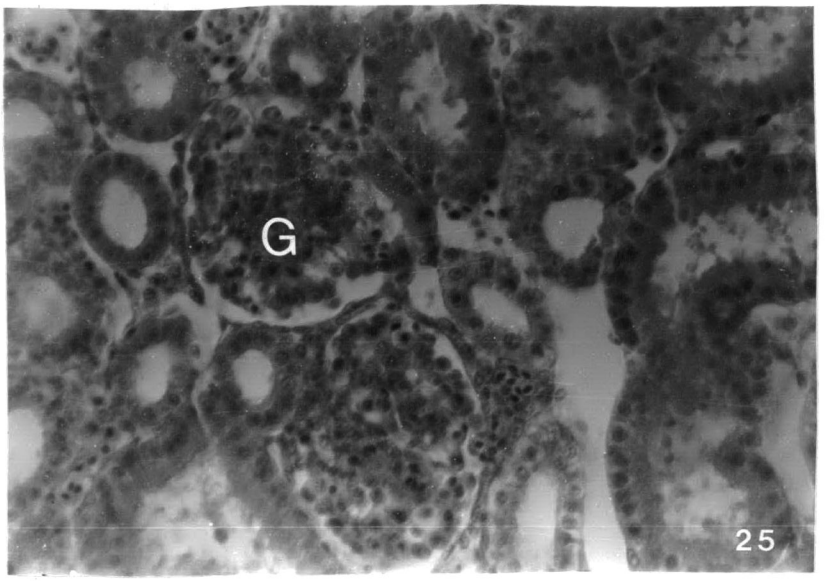

24

Fig. 25. Cross section through the mesonephros of 7-day control embryo showing well preserved glomerulus (G). 45X.

Fig. 26. Cross section through the mesonephros of 7-day Dispersion Yellow 3-treated embryo showing destruction of the glomerulus (G). 10X.


G
25

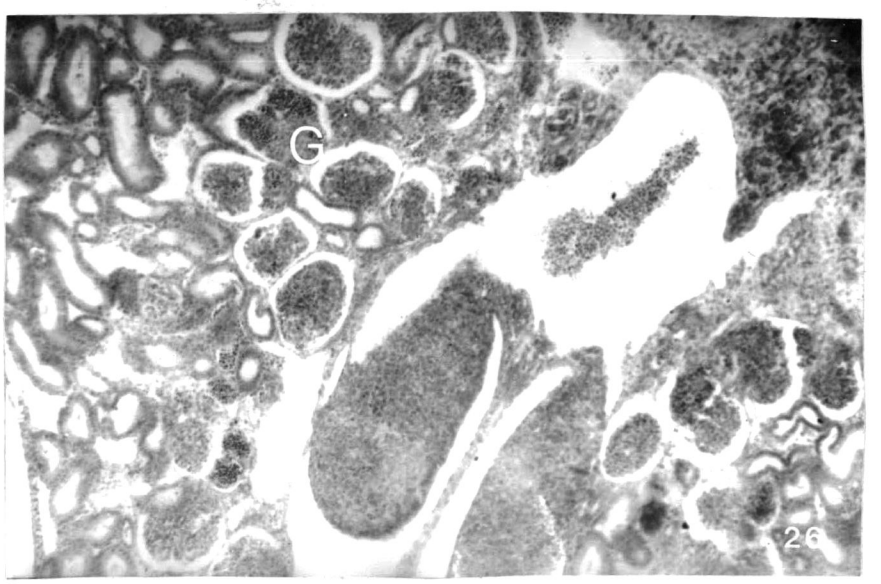
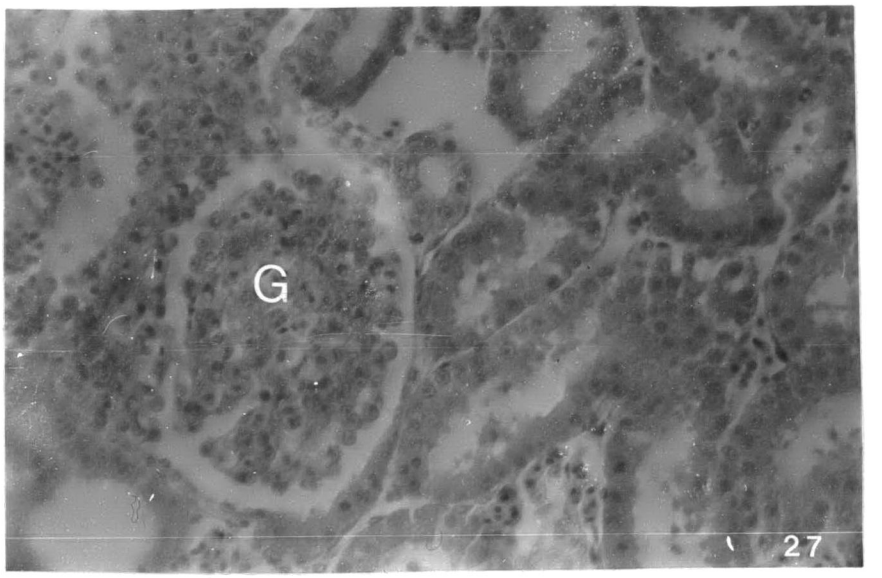

G
26

Fig. 27. Higher magnification of 7-day control embryo showing well preserved glomerulus (G) through the mesonephros. Note intact tubular structures. 45X.

Fig. 28. Higher magnification of 7-day Dispersion Yellow 3-treated embryo showing destruction of the glomeruli (G) through the mesonephros. 45X.


G
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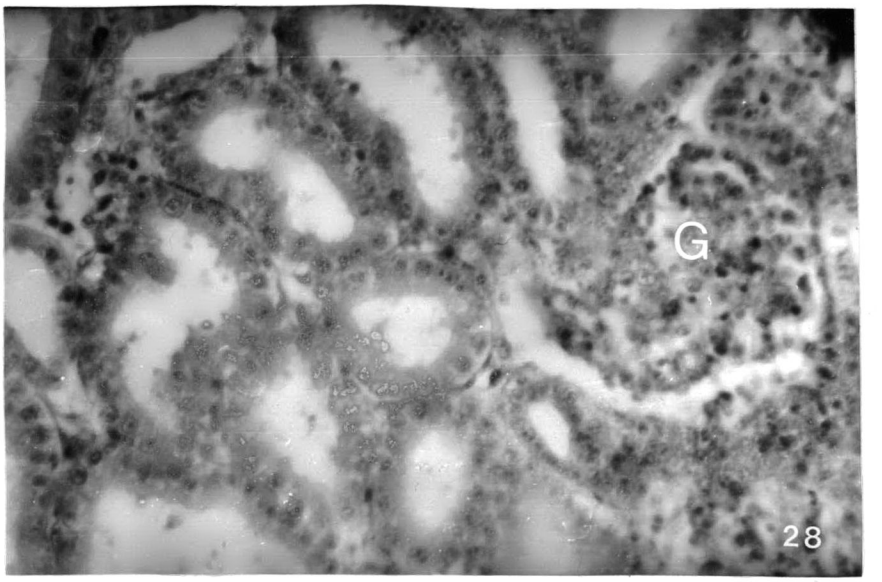
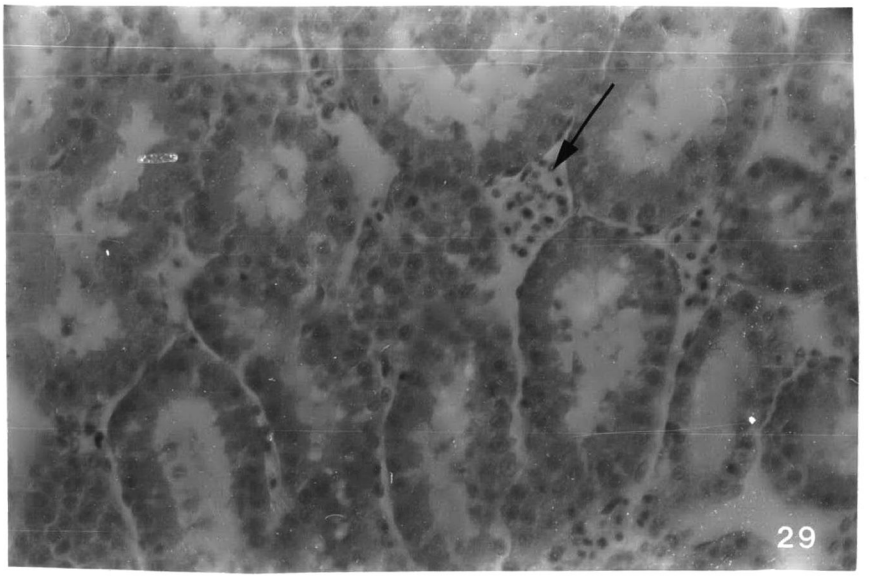

G
28

Fig. 29. Cross section of 8-day control embryo showing absence of ruptured kidney tubules. However, note presence of blood vessels among tubular structures. 45X.

Fig. 30. Cross section of 8-day Dispersion Yellow 3-treated embryo showing mesonephros having a portion of tubular (arrow) structure ruptured. Note disrupted blood vessels are distributed among the tubular structures. 45X.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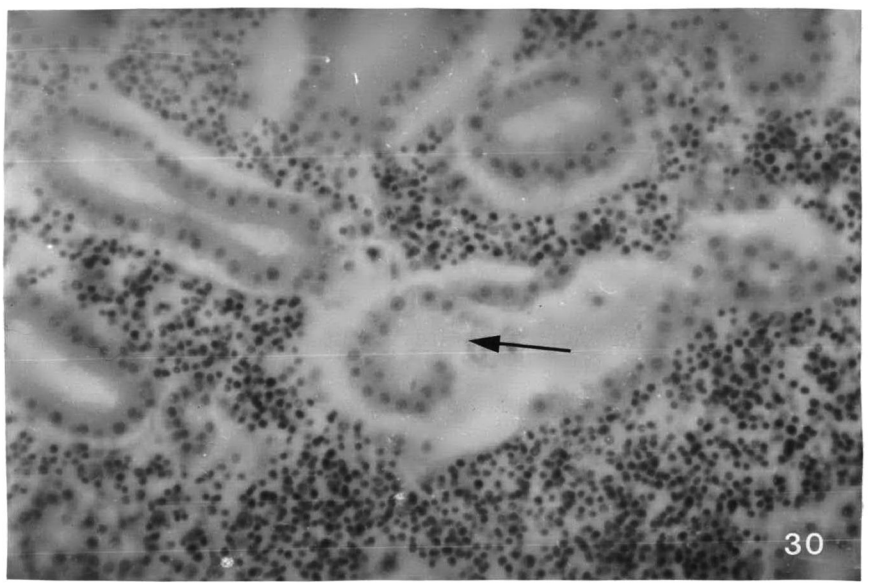
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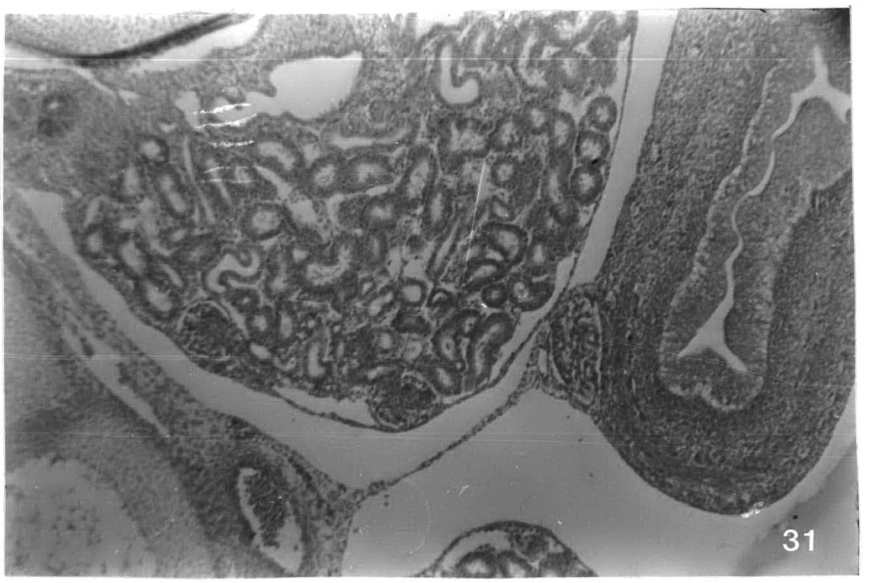
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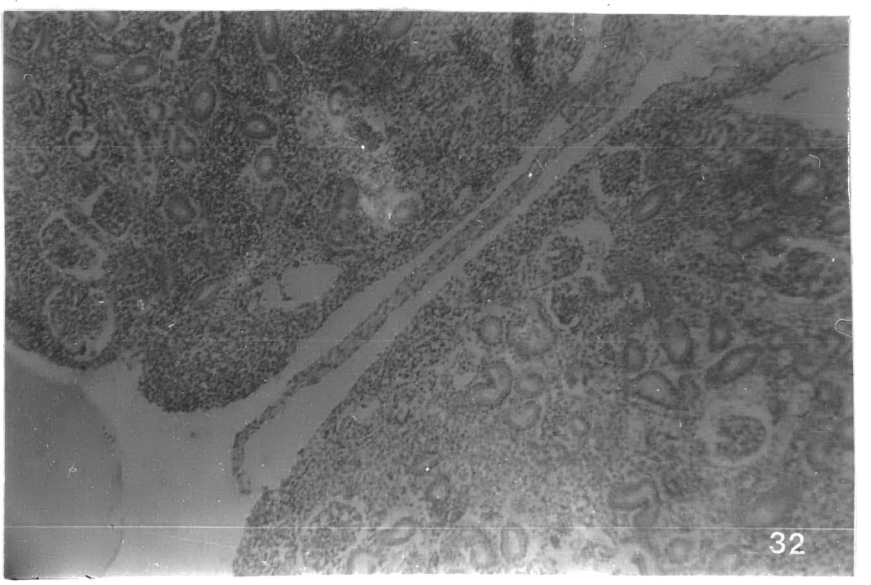
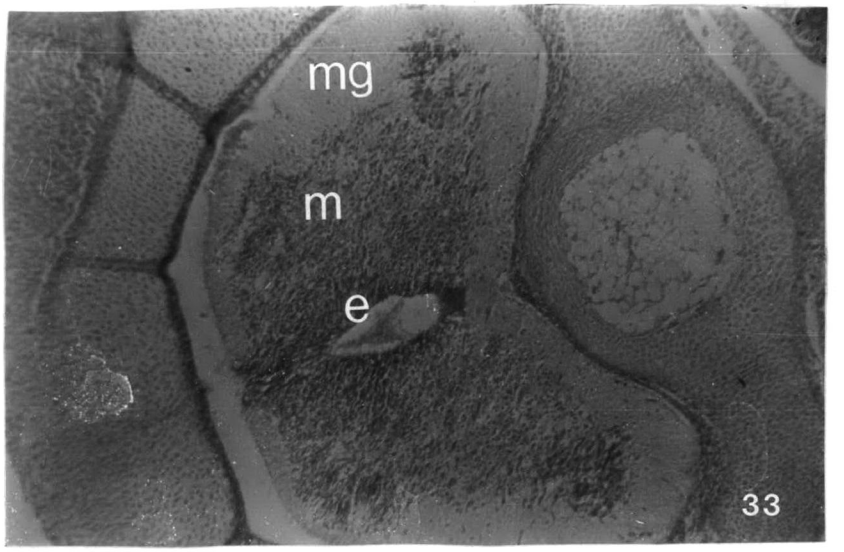
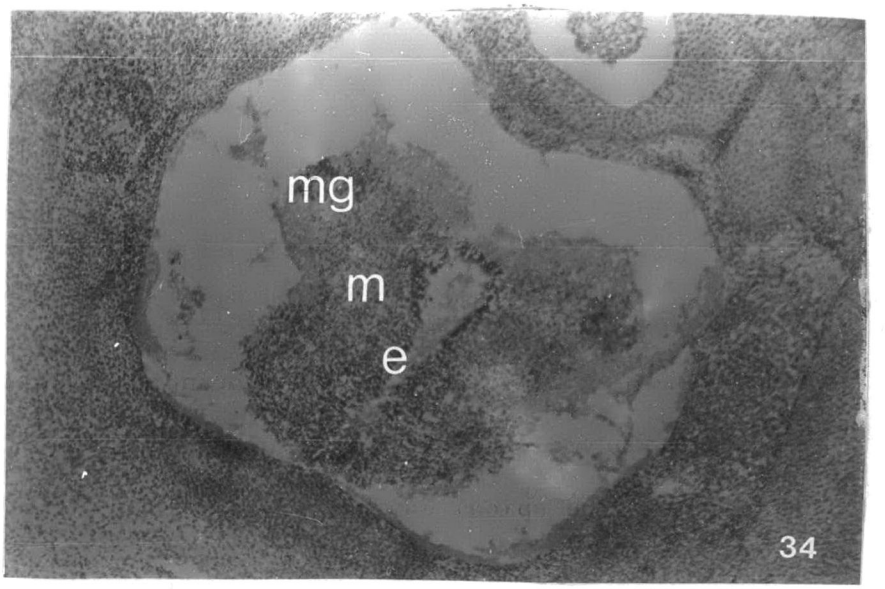

32

Fig. 33. Cross section of 8-day control embryo. Note continuity of the layers (ependymal-e, mantle-m, and marginal-mg) surrounding the spinal cavity. 10X.

Fig. 34. Cross section of 8-day Dispersion Yellow 3-treated embryo showing distortion in the continuity of the layers (ependymal-e, mantle-m, and marginal-mg) surrounding the spinal cavity. 10X.


mg
m
e
33


mg
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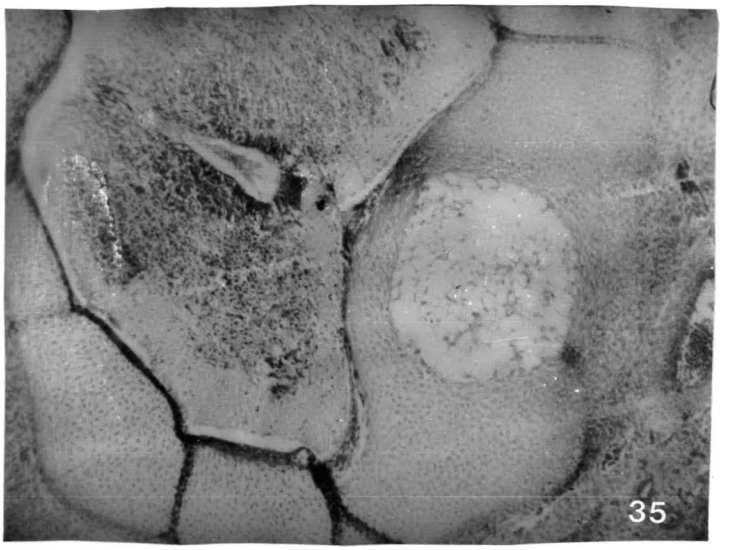
e
34

Fig. 35. Cross section of 10-day control embryo. 10X.

Fig. 36. Cross section through 10-day Dispersion Yellow 3-treated embryo showing most mesonephric tubules (T) destroyed or disarranged (arrow). Note hemorrhaging among tubular structures. 10X.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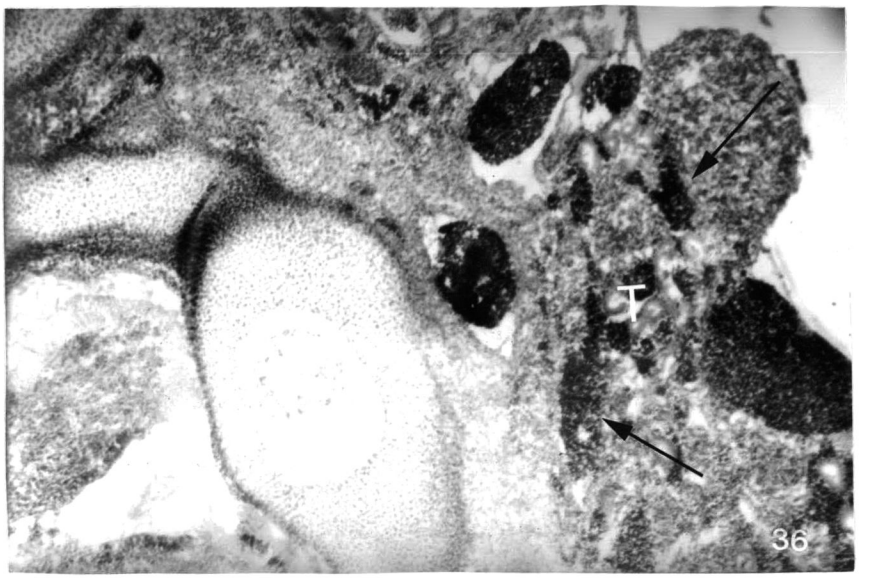
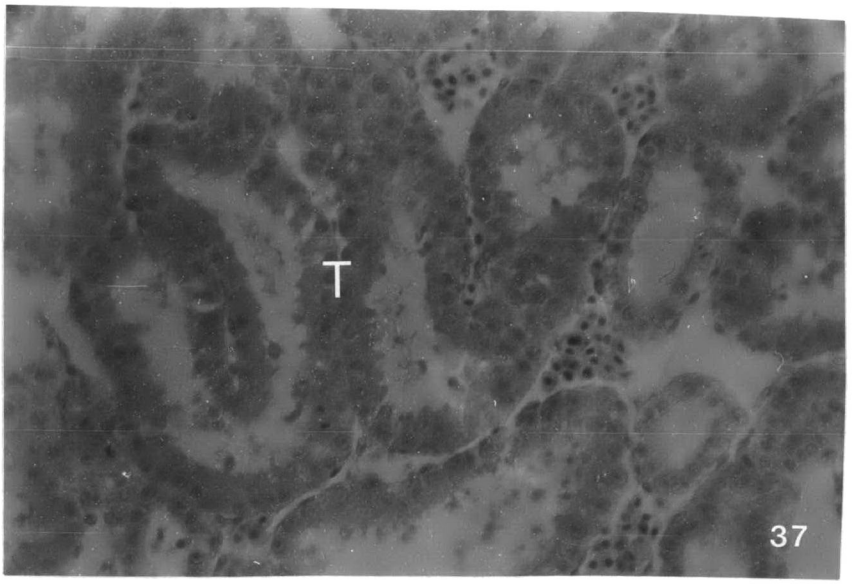

T
36

Fig. 37. Cross section of 10-day control embryo showing intact tubular (T) structures. 45X.

Fig. 38. Cross section of 10-day Dispersion Yellow 3-treated embryo showing ruptured kidney tubules (arrows). Note hemorrhaging among kidney tubules. 45X.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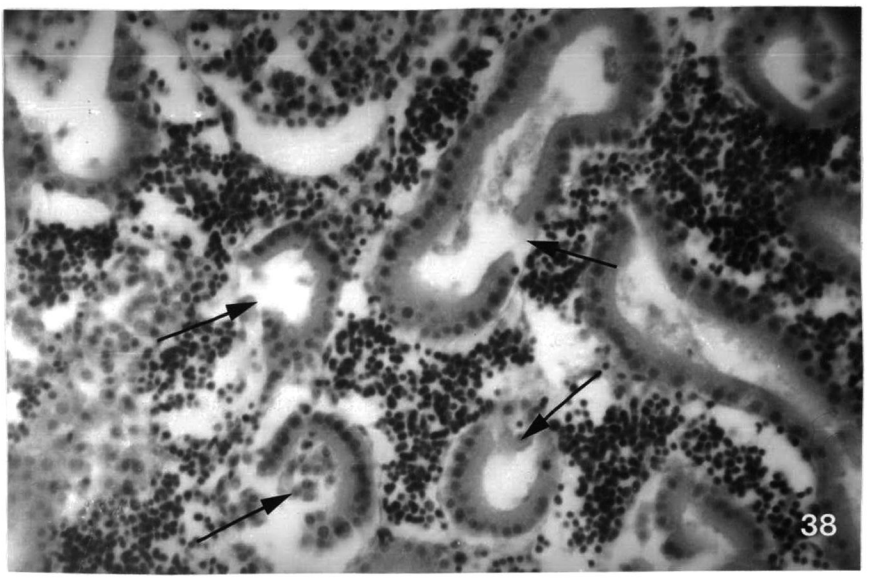
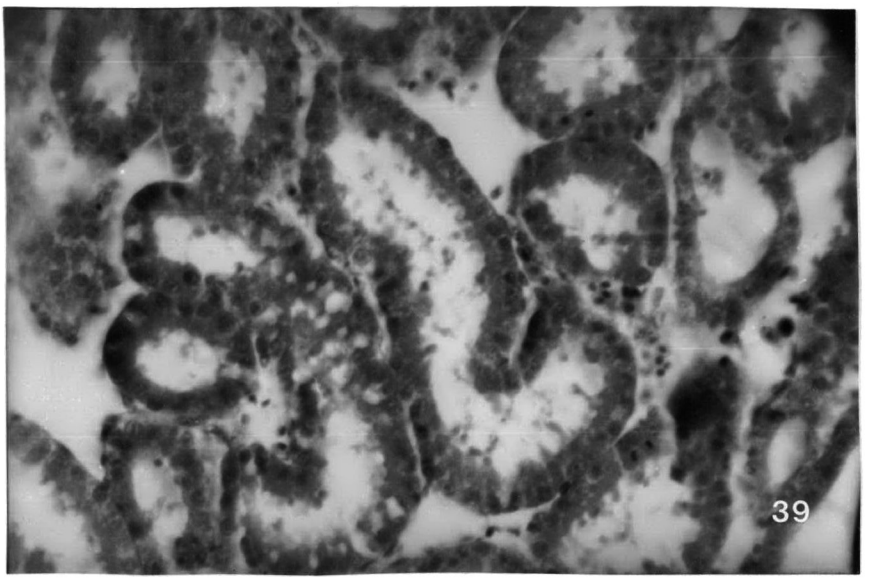

38

Fig. 39. Cross section through mesonephric kidney of 10-day control embryo.

Fig. 40. Cross section of 10-day Dispersion Yellow 3-treated embryo showing a portion of the tubular (T) structure ruptured. Note ruptured blood vessels among tubular structures. 45X.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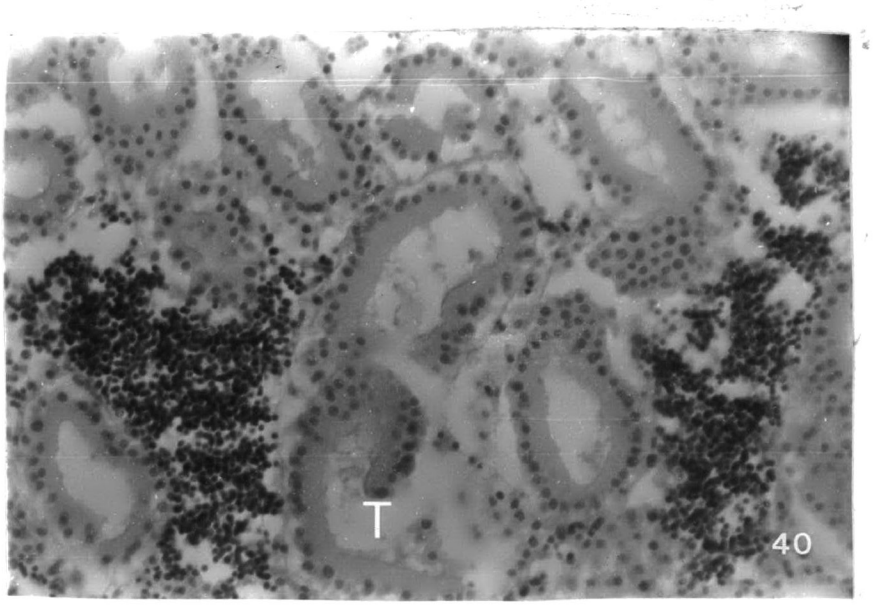
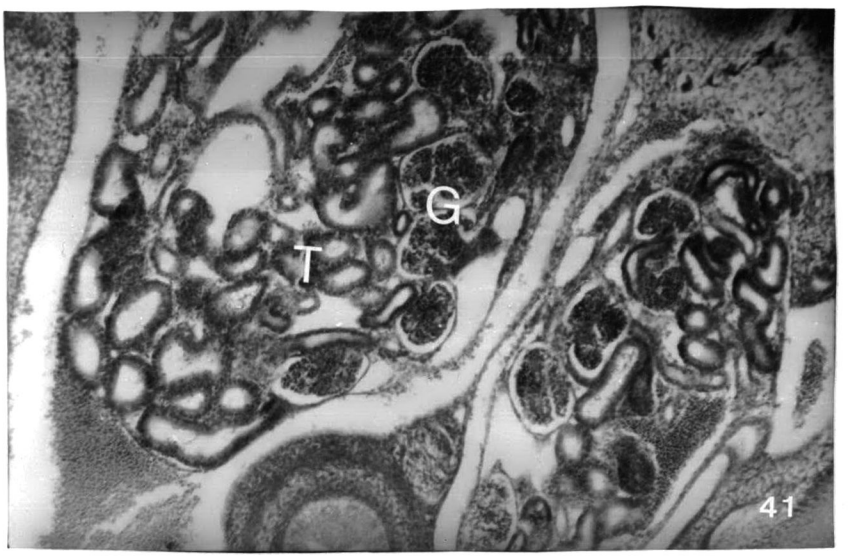

T
40

Fig. 41. Cross section through mesonephros of 10-day control embryo showing well preserved glomerulus (G) and intact tubular (T) structures. 10X.

Fig. 42. Cross section through mesonephros of 10-day Dispersion Yellow 3-treated embryo showing packed blood vessels prominent on one side. Note ruptured tubular (T) structures. 10X.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41

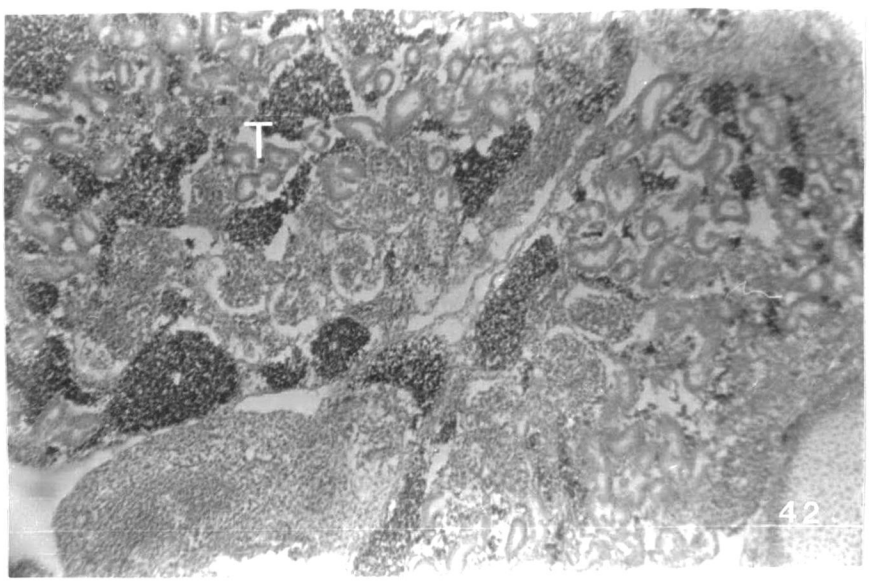
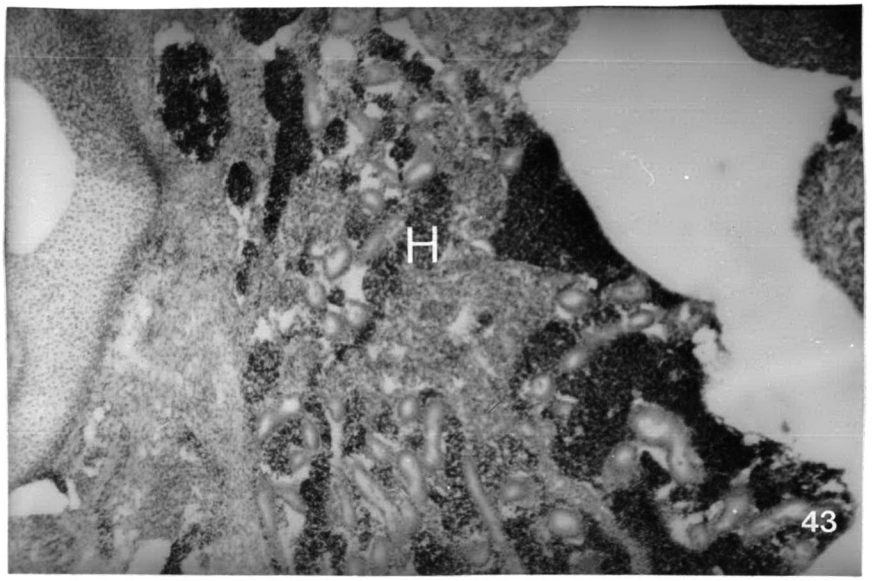
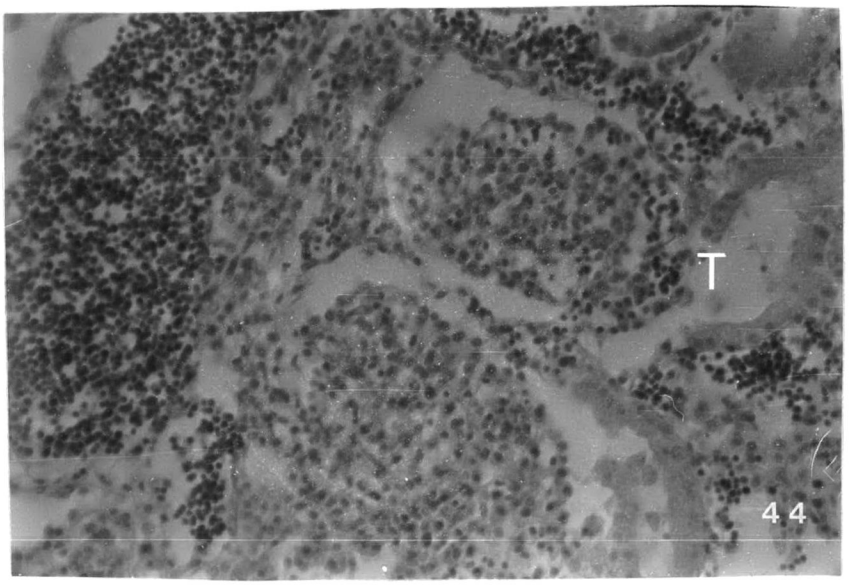

T
42

Fig. 43. Cross section of 10-day Dispersion Yellow 3-treated embryo showing hemorrhagic (H) masses in the mesonephros. 10X.

Fig. 44. Cross section of 10-day Dispersion Yellow 3-treated embryo showing ruptured tubular (T) structures and blood vessels. 45X.


H
43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CHAPTER V

DISCUSSION

Malformations in Dispersion Yellow 3-treated embryos consisted of hemorrhaging, growth retardation, micropthalmia, anopthalmia, exposed viscera and deficient beak. The frequency and types of gross malformations within all the groups did not vary much, except for hemorrhaging. Hemorrhaging was prominent throughout the embryo's body and the most common anomaly observed in this study.

Similar malformations have been reported by previous investigators on teratogenic effects of several environmental pollutants. Wright (1977) observed that the carpet dye Acid Yellow 135 produced hemorrhaging (neck, viscera, eye), growth retardation, micropthalmia, anopthalmia, exposed viscera and feather inhibition. Brain hemorrhaging was the most frequent anomaly observed by Cherry (1976) in his studies using lead acetate; however, dwarfism and micropthalmia also occurred. Callaway (1976) through her observations of the industrial dye trypan blue reported growth retardation and micropthalmia. Gillman et al. (1948) reported similar malformation in rats, mice, and amphibian embryos.

The incidence of eye deformities and exposed visceral organs in DY 3-treated chick embryos was less than the incidence reported using AY 135 by Wright (1977) and Harvey (1979) in chick embryos. Although there is no evidence that azo dyes differ materially in the types of malformations produced, Wilson (1955) suggested that different organs may possess different teratogenic thresholds and that, consequently, those with a high threshold may be only infrequently affected by dyes of low teratogenic potency.

To ascertain whether the action of Dispersion Yellow 3 on chicken embryos was limited to any particular embryonic stage, experiments were performed which showed that the critical period during which the embryos of the developing chicken eggs are sensitive to DY 3 extends from 6 through 12. This period is similar to the developmental stages of 6-10 days that Wright (1977) observed as showing the greatest teratogenic activity in chick embryos exposed to Acid Yellow 135. Callaway (1976) suggested 7-9 days as being the peak of teratogenic activity in such embryos exposed to trypan blue.

Administration of Dispersion Yellow 3 to chicken embryos resulted in ruptured tubular structures and blood vessels, hemorrhaging in mesonephric tubules, destruction of the glomeruli, and distortion in the continuity of the layers (ependymal, mantle, and marginal) surrounding the spinal cavity. The blood vessels among the kidney tubules and ruptured kidney tubules that were observed in the experimental but not in the control, suggested that DY 3 caused damage or swelling in the developmental stages of 8- and 10-day embryos. This agrees with the observations of Harris (1978), although she used cadmium as the teratogen. She reported that cadmium caused pronounced damage to the renal tubular structures. There was a slight swelling of epithelial cells and ruptured blood vessels of the mesonephros which were recognized in earlier stages with dosage continuing to increase with post-incubation time. Histological examination of mesonephric changes revealed degeneration of the glomeruli. The distinct space that is normally between the glomerulus and Bowman's capsule became filled with dispersed cells as a result of damage to the glomeruli.

According to Axelsson and Piscator (1966) and Itokawa et al. (1974), histopathological investigations have demonstrated that renal hypertrophy and degenerative changes involving both the renal tubules and the glomeruli were found after inorganic cadmium intoxication. Damages to the renal tubules were found to be localized mainly in the proximal convoluted tubules while the distal convoluted tubules remained relatively intact. The lesions in the renal glomeruli were usually mild and were only observed in prolonged intoxicated conditions (Axelsson and Piscator, 1966; Flick et al., 1971; Itokawa et al., 1974).

It has been indicated in recent studies using Acid Yellow 135 (Wright, 1977), trypan blue (Callaway, 1976), lead acetate (Cherry, 1976), and cadmium (Harris, 1978) that all of these environmental pollutants work in a similar manner in producing abnormalities. From this investigation, it can be concluded that Dispersion Yellow 3 is teratogenic. Indeed, it is apparent that some dyes, at least, induce a characteristic effect on the embryo when given at the proper period of development.

CHAPTER VI

SUMMARY AND CONCLUSIONS

1. Dispersion Yellow 3 is capable of teratogenic action on chick embryonic development.
2. A total of 758 eggs was utilized in this investigation, with 330 representing the control and 428 representing the experimental.
3. Injections of Dispersion Yellow 3 (DY 3) were given to an experimental series of 48 hr chick embryos.
4. Embryos were sacrificed after 6-12 days of incubation and examined thoroughly for the number of gross abnormalities and the types of malformations produced.
5. Dispersion Yellow 3 produced many gross abnormalities such as hemorrhaging, anopthalmia, micropthalmia, stunted growth, exposed visceral organs and malformation of beaks.
6. Histological analysis showed that the kidney of the developing embryo was the most severely affected by the Dispersion Yellow 3 treatment. Ruptured blood vessels, discontinuity in mesonephric tubules, hemorrhaging in the mesonephros and destruction of the glomeruli were observed. In addition to the other malformations, distortion in the continuity of the layers (ependymal, mantle, and marginal) surrounding the spinal cavity was also noted.

LITERATURE CITED

Auerbach, C. 1949. Chemical mutagenesis. Biol. Rev. (Cambridge). 24:355-391.

_____ 1973. History of research on chemical mutagenesis. pp. 1-19. In A. Hollander, (ed). Chemical mutagens. Plenum Press, New York.

Andrews, W. H. H. and I. del Rio Lozano. 1961. Protein binding and the excretion of some azo dyes in rat bile. Q. J. Exp. Physiol. 46: 238-256.

Axelsson, B. and M. Piscator. 1966. Renal damage after prolonged exposure to cadmium. Arch. Environ. Health 12:360-373.

Beaudoin, A. R. 1961. Teratogenic activity of several closely related disazo dyes on the developing chick embryo. J. Embryol. Exp. Morphol. 9:14-21.

Bender, J. and R. Sheehy. 1976. First Quarterly EPA Report. Grant No. #-804204-1.

Bender, J. 1977. Studies on mutagenicity of chemical carcinogens: A microbial test system. Ph. D. Dissertation, Atlanta University.

Bryan, G. W. 1976. Some aspects of heavy metal tolerance in aquatic organisms. pp. 193. In A. P. M. Lockwood, (ed). Effects of pollutants on aquatic organisms. Cambridge: Cambridge University Press.

Callaway, G. P. 1976. Some teratogenic effects of trypan blue and its components on developing chicken embryos. M. S. Thesis, Atlanta University.

Cardwell, R. D., G. F. Dallas, T. R. Payne, and D. J. Wilbur. 1976. Acute toxicity of selected toxicants to six species of fish.

EPA-600/3-76-008. Contract No. 68-01-0748. U. S. Environmental Protection Agency-Environmental Research Lab, Duluth, Minnesota.

Catizone, O. and P. Gray. 1941. Experiments on chemical interference with the early morphogenesis of the chick. J. Exp. Zool. 87:71.

Cherry, C., Jr. 1976. A chemical and morphological investigation of some effects of lead acetate on the brain of chick embryo. M. S. Thesis, Atlanta University.

Daoust, R. and A. Cantero. 1959. The numerical proportions of cell types in rat liver during carcinogenesis by 4-dimethylaminoazobenzene (DAB). Cancer Res. 19:757-762.

Davies, P. H. and W. H. Everhart. 1973. Effects of chemical variations in aquatic environments. Vol. III. Lead toxicity to rainbow trout and testing application factor concept. EPA-R3-73-011c. Proj. 18050 DYC. Colorado State University, Fort Collins, Co.

EPA Report. 1974. "Organic characterization study of Coosa River Basin-Northwest Georgia." U. S. Environmental Protection Agency, Region IV, Surveillance and Analysis Division, Athens, Georgia.

Fare, G. 1966. Rat skin carcinogenesis by tropical applications of some azo dyes. Cancer Res. 26:2406-2408.

Flick, D. F., H. F. Kraybill, and J. M. Dimitroff. 1977. Toxic effects of cadmium: A review. Environ. Res. 4:71-85.

Fromme, P. O. and R. M. Stokes. 1962. Assimilation and metabolism of chromium by trout. Water Pollut. Cont. Fed. J. 34:1151-1155.

Gilbertson, M. and G. A. Fox. 1977. Pollutant associated embryonic mortality of Great Lakes herring gulls. Environ. Pollut. 12:211-215.

Gillman, J., C. Gilbert, and T. Gillman. 1948. A preliminary report on

hydrocephalus, spina bifida and other congenital anomalies in the rat produced by trypan blue. S. Afr. J. Med. Sci. 13:47-90.

Gordon, J. 1964. Contact reactivity to azo dye carcinogens. Nature 203:884-885.

Gray, P. S. 1978. Effects of Dispersion Yellow 3 on oocyte development in Rana clamitans larvae: A histological, cytochemical, and ultrastructural analysis. Ph. D. Dissertation, Atlanta University.

Gray, P. S., R. Hunter, Jr., and R. M. Patterson. 1979. Chromosomal aberrations induced by Dispersion Yellow 3 in Rana clamitans larvae during tail regeneration. Cytobios 25:175-182.

Harris, S. 1978. Cadmium toxicity in the kidneys and liver of 4-7 day chicken embryos: Histological, histochemical, and atomic spectrophotometric analysis. Ph. D. Dissertation, Atlanta University.

Hamburg, M. 1952. Malformations in mouse embryos induced by trypan blue. Nature 169:27.

_____ 1954. The embryology of trypan blue induced abnormalities in mice. Anat. Rec. 103:409-422.

Harm, H. 1954. Der einfluss von Trypan Blau auf die Nachkommenschaft trachtiger kaninchen. Zeits. F. Naturforschung 9b:536-540.

Harvey, L. S. 1979. The effects of Acid Yellow 135, a water pollutant, on the development of the eye in chick embryos. M. S. Thesis, Atlanta University.

Hogan, A. G., B. S. O'Dell, and J. R. Whitley. 1950. Maternal nutrition and hydrocephalus in newborn rats. Proc. Soc. Exp. Biol. Med. 74:293-296.

Humason, G. L. 1962. Animal tissue techniques. W. H. Freeman and Co.,

San Francisco.

Itokawa, Y., T. Abe, R. Tabel, and S. Tanaka. 1974. Renal and skeletal lesions in experimental cadmium poisoning. Arch. Environ. Health 28:149-154.

Levine, W. T. 1932. The effects of x-rays on the gonads of amphibian larvae. J. Exp. Zool. 62:409-431.

Little, L. W., J. C. Lamb III, M. A. Chillingworth, and W. B. Durkin. 1974. Acute toxicity of selected commercial dyes to the fathead minnow and evaluation of biological treatment for reduction of toxicity. Proceedings of the 29th Industrial Waste Conference. UNC Wastewater Research Center. Department of Environmental Sciences and Engineering-School of Public Health, University of North Carolina, Chapel Hill, NC 27514.

Morrison, R. T. and R. N. Boyd. 1977. Organic Chemistry. 3rd ed. Allyn and Bacon, Inc.

Murakami, U. 1952. Artificial induction of pseudencephaly, short-tail, tail-lessness, myelencephalic blebs and some fissure formations (phenocopies) of the mouse. Nagoya J. Med. Sci. 15:185-194.

Netzer, A., H. K. Miyamoto, and P. Wilkinson. 1975. Heavy metal concentration of exhausted dye bath effluents. Bull. Environ. Contam. Toxicol. 14:301-303.

Neubert, D. 1974. The toxicological evaluation of mutagenic events. Mut. Res. 25:145-157.

Opie, E. L. 1946. Mobilization of basophilic substances (ribonucleic acid) in the cytoplasm of liver cells with the production of tumors by butter yellow. J. Exp. Med. 84:91-106.

Orr, J. W. 1948. Observation on the hepatotoxic action of the carcinogen p-dimethylaminoazobenzene. J. Pathol. Bacteriol. 60:461-469.

Price, J. M., J. W. Harman, E. C. Miller, and J. A. Miller. 1952. Progressive microscopic alterations in the livers of rats fed the hepatic carcinogens 3'-methyl-4-dimethyl-aminoazobenzene and 4'-fluoro-4-dimethylaminoazobenzene. Cancer Res. 12:192-200.

Rothstein, A. 1959. Cell membrane as the site of action of heavy metals. Fed. Proc. 18:1026-1035.

Singh, S. M. and P. N. Ferns. 1978. Accumulation of heavy metals in rainbow trout, Salmo gairdneri (Richardson), maintained on a diet containing activated sewage sludge. J. Fish Biol. 13:277-286.

Tincher, W. C. 1973. Effects of polyester fiber processing effluents on water quality. Atlanta: School of Textile Engineering in Cooperation with Environmental Resources Center, Georgia Institute of Technology. 51 L. Illus. ERC-1673. Completion Report, OWRR Project No. A-043-GA.

_____ 1974. Chemical use and discharge in carpet piece dyeing. Project Report #-27-626. Environmental Protection Division. Dept. of Natural Resources, Atlanta, Georgia. Sept.

_____ 1974. Identification and qualification of organic contaminants from textile processing. Wayne C. Tincher. Atlanta: School of Textile Engineering Georgia Institute of Technology. 197401975. 12V. IN 1:ILL; 28 CM. Report No. 1-10 and Final Report, Project E-27-626.

_____ 1975. Survey of Coosa River Basin for organic contaminants from carpet processing. Atlanta: School of Textile Engineering,

Georgia Institute of Technology. 30V. IN 1: ILL.; 29 CM. Report Nos. 1-29 and Final Report, Project E-27-630.

Waddington, C. H. and T. C. Carter. 1952. Malformations in mouse embryos induced by trypan blue. Nature 169:27-28.

Waddington, C. H. and M. Perry. 1956. Teratogenic effects of trypan blue on amphibian embryos. J. Embryol. Morphol. 4:110-118.

Watters, C. and A. Cantero. 1965. Rat liver parenchymal cell function during azo dye carcinogenesis. Cancer Res. 25:67-70.

Wilson, J. G. 1954. Withdrawal of claim that azo blue causes congenital malformations. Proc. Soc. Exp. Biol. Med. 87:1.

Wilson, J. 1955. Teratogenic activity of several azo dyes chemically related to trypan blue. Anat. Rec. 123:313-326.

Wright, C. 1977. The effects of Acid Yellow 135, a water pollutant, on the development of chick embryos. M. S. Thesis, Atlanta University.

Yoshida, T. 1933. Uber die serienweise Verfolgung der Veranderungen der Leber bei der experimentellen Hepatomerzeugung durch O-amidoazotoluol. Trans. Jap. Path. Soc. 23:636-638.